Vitamin D Receptor Polymorphisms and Prostate Cancer

Dan G. Blazer III,[1] David M. Umbach,[2] Roberd M. Bostick,[3] and Jack A. Taylor[1,4]*

[1]*Laboratory of Molecular Carcinogenesis*
[2]*Biostatistics Branch*
[3]*Department of Public Health Sciences—Epidemiology, Wake Forest University School of Medicine, Wake Forest University, Winston-Salem, North Carolina*
[4]*Epidemiology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina*

Prostate cancer is a common disease, yet determinants of prostate cancer risk remain largely unidentified. Low circulating levels of 1,25-dihydroxy vitamin D (1,25-D) have been implicated as a risk factor for prostate cancer. In addition, 1,25-D exhibits significant antineoplastic properties both in vitro and in vivo, and these antiproliferative effects appear to be mediated through the vitamin D receptor (VDR). The VDR has a number of common polymorphisms, including a TaqI restriction fragment length polymorphism in exon 9 and a poly(A) length polymorphism in the 3′-untranslated region. Previous studies have found an association between the TaqI T allele or poly(A) L allele and prostate cancer. To further investigate the putative link between VDR polymorphisms and prostate cancer, we conducted a case-control study of prostate cancer patients from the Piedmont region of North Carolina. Using polymerase chain reaction–based techniques on DNA extracted from peripheral blood, we genotyped 77 cases (70 white, seven black) and 183 controls (169 white, 14 black) for the TaqI and poly(A) alleles. We report here an overall lack of association between either the TaqI or poly(A) genotype and prostate cancer odds ratio (OR) = 1.4, 95% confidence interval (CI) = 0.7–2.8; and OR = 1.2, 95% CI = 0.6–2.5, respectively). Using a case–case analysis, we tested whether these polymorphisms might be associated with more advanced disease but found no statistically significant association for the TaqI T or poly(A) L allele (OR = 2.5, 95% CI = 0.3–21.7; OR = 2.8, 95% CI = 0.3–23.8, respectively). We report strong evidence of linkage disequilibrium between the TaqI and poly(A) polymorphisms ($P < 0.0001$), with whites demonstrating stronger linkage disequilibrium than blacks (D = 0.24 vs. D = 0.18). *Mol. Carcinog. 27:18–23, 2000.* © 2000 Wiley-Liss, Inc.

Key words: vitamin D receptor; prostate cancer; polymorphism; epidemiology; vitamin D

INTRODUCTION

Prostate cancer is the second most common malignancy in men after skin cancer and the second most common cause of cancer death [1]. About 184 500 new cases of prostate cancer are expected in the United States in 1998, accounting for 29% of all new cancer cases in men [1]. Prostate cancer is expected to result in 39 200 deaths, accounting for 13% of all cancer deaths and killing nearly as many men as breast cancer kills women [1]. Despite the prevalence of and mortality associated with this disease, risk factors other than age, race, and geographic location remain largely unknown.

Vitamin D deficiency has been hypothesized to be a risk factor for prostate cancer [2]. Reduced vitamin D levels correlate with established risk factors such as increasing age, African-American race, and residence in northern latitudes [2]. Vitamin D and vitamin D analogs demonstrate significant antineoplastic properties in vitro [3–7] and may even reduce metastatic potential of prostate cancer cells [8,9]. In a population-based study, Corder et al. [10] used prediagnostic serum samples to show that risk of prostate cancer decreased with higher levels of 1,25-dihydroxy vitamin D (1,25-D), the biologically active metabolite of vitamin D, although this finding has not been replicated in subsequent studies [11,12]. In response to these findings, a pilot study examining the effects of 1,25-D administration to patients with early recurrent prostate cancer has already been launched [13].

The antineoplastic actions of vitamin D appear to be mediated primarily through the vitamin D receptor (VDR) [14–16]. The VDR, a member of the steroid/thyroid hormone nuclear receptor superfamily, is expressed in both normal and cancerous prostate cells [3,4,7,17]. 1,25-D binds the VDR, and this complex forms a heterodimer with the retinoid X receptor. This heterodimer in turn binds vitamin D response elements on DNA and regulates tran-

The present address of Roberd M. Bostick is South Carolina Cancer Center, University of South Carolina, Columbia, SC 29425.

*Correspondence to: National Institute of Environmental Health Sciences, 111 T.W. Alexander Dr., P.O. Box 12233, MD A3-05, Research Triangle Park, NC 27709.

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Abbreviations: 1,25-D, 1,25-dihydroxy vitamin D; VDR, vitamin D receptor; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; 3′-UTR, 3′ untranslated region; OR, odds ratio; CI, confidence interval.

scription of numerous genes, including *p21*, *p27*, c-*fos*, and c-*myc*, which are all involved in cell growth and differentiation [18].

Polymorphisms in the VDR could alter receptor function and affect prostate cancer susceptibility. Common polymorphisms in the VDR were first identified in studies related to bone metabolism and bone mineral density [19,20]. These polymorphisms include two restriction fragment length polymorphisms (RFLPs) in intron 8 of the VDR—BsmI and ApaI—and a TaqI RFLP at codon 352 in the last exon, exon 9, which is ''silent,'' i.e., it does not result in an amino acid change. In addition, Morrison et al. [20] identified numerous polymorphisms in the 3′ untranslated region (3′-UTR) of the VDR, including a variable length mononucleotide run of adenines that is 5′ to the polyadenylation signal. While the RFLPs in intron 8 and exon 9 are unlikely to have functional significance, there is some evidence that sequence differences in the 3′-UTR may affect transcription [20]. There is also some evidence that the RFLPs in intron 8 and exon 9 may be in linkage disequilibrium with polymorphisms in the 3′-UTR [21].

Our laboratory first reported an association between the TaqI T allele and an increased risk of prostate cancer [22]. Ingles et al. [23] subsequently reported an increased risk of prostate cancer associated with the poly(A) L allele and that the L allele appears to confer risk for more advanced disease. Some indirect evidence suggests that these TaqI and poly(A) alleles may be in linkage disequilibrium in Caucasians [19–21], but this has not been tested directly to date.

We report here the results of a case-control study investigating the association of TaqI and poly(A) genotypes and prostate cancer risk. In addition, we investigate the strength of linkage disequilibrium between these two loci.

SUBJECTS AND METHODS

Subjects

Our study involved participants in the Markers of Prostate Cancer study, a community-based case-control study in the Piedmont region of North Carolina conducted out of the Wake Forest University School of Medicine. All participants were aged 50 yr or older and were residents of the Piedmont Triad metropolitan area. Participants were excluded if they had a history of a previous cancer (other than nonmelanoma skin cancer), current prostate disease (e.g., symptomatic benign prostatic hypertrophy or prostatitis), previous prostate surgery, active tuberculosis, current liver or kidney disease, or intolerance to caffeine or cough syrup. Eligibility for cases also included that participants had a biopsy-proven first diagnosis of incident prostate cancer (any stage or grade).

Cases (n = 112) were identified through area urology and radiation oncology practices within days of diagnosis and were studied prior to treatment. Thus, all cases are incident cases. Controls (n = 258) were randomly selected from the Piedmont Triad community and were frequency matched with cases on age (5-yr intervals), race, and zip code. Participants were accrued from February 1994 through January 1996. Blood samples for DNA genotyping were obtained only on the last 260 participants (77 cases and 183 controls).

TaqI Genotyping

VDR genotype with respect to the TaqI RFLP in exon 9 was determined as described previously [22,24]. Briefly, a 740-bp fragment was amplified by polymerase chain reaction (PCR) from genomic DNA by using the forward primer 5′CAGAGCATGGACAGGGAGCAA3′ and the reverse primer 5′GCAACTCCTCATGGCTGAGGTCTC3′. Amplified products were then subjected to TaqI digestion and run on 3% Nusieve 3:1 agarose gels (FMC Bioproducts, Rockland ME). Alleles were scored for presence (t) or absence (T) of the polymorphic TaqI restriction endonuclease site. The three resulting genotypes were designated TT, Tt, and tt.

Poly(A) Polymorphism Genotyping

The VDR poly(A) length polymorphism in the 3′-UTR was analyzed by first amplifying by PCR a 196-bp fragment from genomic DNA by using the forward primer 5′CTAGAAGTGGGCCAGGACAG3′ and the reverse primer 5′TACAGGCTTGCGCCACCATG3′. Approximately 25 ng of genomic DNA was amplified in the following 20-μL reaction mixture: 2 μL of 10× PCR Buffer II containing 15 mM $MgCl_2$, 1 μM each primer, 1 U of AmpliTaq Gold DNA polymerase, 0.2 mM dNTPs, and 0.2 mL of [^{33}P] dATP. All PCR reagents were supplied by PE Applied Biosystems (Foster City, CA) except [^{33}P] dATP (Amersham Corporation, Arlington Heights, IL). Cycling conditions were 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s. All reactions were performed in a GeneAmp PCR System 9700 thermocycler (PE Applied Biosystems). PCR products were then separated on a 6% polyacrylamide sequencing gel for 4.5 h at 80 W and autoradiographed. Allele sizes were compared against one another and against known controls. Alleles resolved into two distinct sizes and were designated ''short'' or ''long'' [23]. The resulting genotypes were SS, LL, and SL (see Figure 1).

Tumor Staging

Information on prostate cancer pathology and staging was obtained through the North Carolina Central Tumor Registry. Tumors were staged by the TNM classification system. Local disease was defined

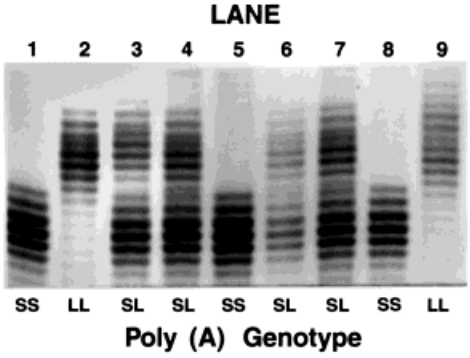

Figure 1. Determination of three possible VDR poly(A) genotypes by polyacrylamide gel electrophoresis.

as prostate cancer confined within the prostate. Advanced disease was defined as prostate cancer that extended through the prostate capsule, with fixation or invasion of adjacent structures, involvement of regional lymph nodes, and/or demonstrated evidence of metastasis. Tumor stage was unknown for 6 of the 77 cases (six white, 0 black), and therefore, these cases were excluded from analysis involving stage of disease. Independent pathology examination of tumor specimens was not conducted.

Statistical Analysis

We evaluated linkage disequilibrium between the TaqI and poly(A) alleles by using well-known likelihood methods [25]. Since our methodology for genotyping cannot assign haplotypes to doubly heterozygous individuals (Tt/SL genotypes), we employed the EM algorithm to maximize the multinomial likelihood in the face of that indeterminacy under the assumption that our samples represented a randomly mating population [25,26]. We programmed the likelihood calculations in GAUSS (Aptech Systems, Maple Valley, WA).

For crude analyses of the relationship of genotype to disease risk or to tumor aggressiveness, we calculated odds ratios (ORs) and confidence intervals (CIs) by using standard methods or exact methods when sample sizes warranted [27]. For analyses that also adjusted for age, race, or other factors, we used logistic regression methods [28]. Age adjustments were based on either continuous age terms (linear and quadratic) or on a median split of the distribution of age at diagnosis. Our study included two persons (one case, one control) who classified themselves as other than black or white; in our analyses, we grouped these individuals with whites.

We employed SAS software (SAS Institute, Cary, NC) and StatXact (Cytel Statistical Software, Cambridge, MA) for statistical calculations. We report *P* values and CIs based on Wald statistics; all *P* values are for two-tailed tests.

RESULTS

Using the PCR-based techniques described above, we genotyped 77 cases (70 white, seven black) and 183 controls (169 white, 14 black) for the TaqI and poly(A) alleles.

Linkage Disequilibrium Between TaqI and Poly(A) Polymorphisms

Overall, we found strong evidence of linkage disequilibrium ($P < 0.0001$) between the TaqI and poly(A) alleles, with a calculated disequilibrium coefficient (D) of 0.24. The concordant haplotypes—TL and tS—totalled 98% of all haplotypes (see Table 1). The disequilibrium coefficient for both cases and controls considered individually was also 0.24. Linkage disequilibrium was most complete among our white subjects, where TL and tS haplotype frequencies totalled 99% (Table 1). Among blacks, the strength of linkage disequilibrium was still highly significant ($P < 0.0001$) but weaker than among whites ($D = 0.16$, TL and tS haplotype frequencies totalled 87.5%). The racial difference in disequilibrium coefficient was statistically significant ($P = 0.015$).

TaqI and Poly(A) Polymorphisms and Prostate Cancer Risk

Overall, neither the TaqI T nor poly(A) L allele was associated with increased prostate cancer risk. We compared subjects homozygous and heterozygous for the T or L allele to those homozygous for the t or S allele, while controlling for age and race (see Table 2). The OR for the T genotypes (TT + Tt vs. tt) was 1.4 (95% CI = 0.7–2.8), and the OR for the poly(A) L genotypes (LL + SL vs. SS) was 1.2 (95% CI = 0.6–2.4). Considering whites alone, the results were similar, and there was no association between genotype and disease (Table 2). ORs for blacks alone were not calculated because of the small number of subjects. Additionally, there was no evidence of a ''dose-response'' effect of the TaqI T and poly(A) L alleles.

TaqI and Poly(A) Polymorphisms and Tumor Aggressiveness

Among cases, we evaluated whether the TaqI T or poly(A) L alleles were associated with more advanced disease, i.e., cancer cases not confined to the prostate, by using a case-case comparison. We

Table 1. Correlation between TaqI and Poly(A) Genotypes

	Whites (n = 238)			Blacks (n = 21)		
	TT	Tt	tt	TT	Tt	tt
LL	79	1	0	9	0	1
SL	1	109	0	3	5	0
SS	0	1	47	0	0	3

Table 2. Frequencies of TaqI and Poly(A) Genotypes and ORs of Prostate Cancer According to Genotype

	White (%)		Black (%)		White*			Combined†		
Genotype	Case (n = 70)	Control (n = 169)‡	Case (n = 7)	Control (n = 14)	OR	95% CI	*P*	OR	95% CI	*P*
TaqI										
tt	17%	21%	14%	21%	1§			1§		
Tt	53%	44%	43%	14%	1.5	0.7–3.4	0.27	1.6	0.8–3.4	0.20
TT	30%	35%	43%	64%	1.1	0.5–2.6	0.77	1.1	0.5–2.4	0.83
(TT + Tt)	83%	79%	86%	78%	1.4	0.7–2.9	0.41	1.4	0.7–2.8	0.37
Poly(A)										
SS	19%	21%	14%	14%	1§			1§		
SL	50%	45%	57%	28%	1.3	0.6–2.9	0.45	1.4	0.7–2.9	0.40
LL	31%	34%	29%	57%	1.1	0.5–2.5	0.82	1.0	0.5–2.2	0.98
(LL + SL)	81%	79%	86%	85%	1.2	0.6–2.5	0.56	1.2	0.6–2.4	0.59

*From logistic regression adjusting for age as a continuous variable.
†From logistic regression adjusting for race and for age as a continuous variable.
‡n = 168 for poly(A) white controls.
§Referent.

compared cases homozygous and/or heterozygous for the T or L alleles to those homozygous for the t and S alleles. There was some evidence of increased tumor aggressiveness with the T and L alleles, although this association was not statistically significant (Table 3). The OR of advanced disease for the TaqI T genotypes (TT + Tt vs. tt) was 2.3 (95% CI 0.3–112). The OR of advanced disease for the poly(A) L genotypes (LL + SL vs. SS) was 2.5 (95% CI 0.3–120).

DISCUSSION

Several common polymorphisms in the VDR have been identified, including RFLPs in intron 8, a ''silent'' TaqI RFLP in exon 9, and several in the 3′-UTR. The commonly studied RFLPs are unlikely to have direct functional consequences, but they may be markers of a nearby functional polymorphism within the VDR gene or a nearby gene [29].

We found the TaqI and poly(A) alleles of the VDR to be in linkage disequilibrium. The relationship was stronger in whites than blacks. These results are supported by previous studies showing linkage disequilibrium between BsmI and TaqI alleles [29] and BsmI and poly(A) alleles [21]. Our finding that linkage disequilibrium between the TaqI and poly(A) alleles was weaker in the small sample of blacks is consistent with previous work demonstrating ethnic differences in linkage disequilibrium of VDR polymorphisms [21].

We find little evidence for an association between VDR TaqI or poly(A) genotypes and prostate cancer risk (TT + Tt vs. tt: OR = 1.4, 95% CI = 0.7–2.8; LL + SL vs. SS: OR = 1.2, 95% CI = 0.6–2.4). Our

Table 3. Association of Genotype with Disease Stage

	Stage (no.)				
VDR polymorphism	Advanced	Local	OR*	95% CI*	*P**
TaqI					
tt	1	11	1†		
Tt	8	28	2.9	0.3–145	0.6
TT	3	20	1.2	0.1–76	1.0
(TT + Tt)	11	48	2.3	0.3–112	0.8
Poly(A)					
SS	1	12	1†		
SL	8	27	3.1	0.3–152	0.5
LL	3	20	1.5	0.1–92	1.0
(LL + SL)	11	47	2.5	0.3–120	0.7

*From exact logistic regression adjusting for race and for age dichotomized at the median.
†Referent.

results differ from two previous reports that found increased risk associated with the TaqI T allele and the poly(A) L allele [22,23]. Taylor et al. [22] reported in a case-control study of 108 cases and 170 controls that the TaqI T allele appear to confer increas risk of prostate cancer (TT + Tt vs. tt: OR = 2.9, 95% CI = 1.3–6.3). In a similar study, Ingles et al. [23] found in a study of 57 cases and 169 controls that the poly(A) L allele is associated with a > 4-fold increase in the risk of prostate cancer (LL + SL vs. SS: OR = 4.61, 95% CI = 1.34–15.82). However, the lack of association reported here is consistent with a larger, more recent nested case-control study (372 cases, 591 controls) by Ma et al. [29] from the Physicians' Health Study, which found no association between TaqI or BsmI polymorphisms and prostate cancer.

The difference in results among these four studies is unlikely to be due to selection of control groups since genotype frequencies among controls are remarkably consistent across all studies. There is a possibility that undiagnosed latent cases of prostate cancer in the control group might lead to misclassification and could bias towards the null. Case selection could play a role: our study and the study by Ma et al. [29] used incident cases, whereas the first studies [22,23] used prevalent cases. However, except for the unlikely situation in which the putative protective VDR alleles are associated with early mortality, inclusion of prevalent cases would tend to bias towards the null. Instead, studies of incident cases have found no association, whereas studies of prevalent cases have found an association.

In light of recent results suggesting that vitamin D has antimetastatic properties in addition to its antiproliferative properties [8,9], we investigated whether the putative at-risk VDR alleles might be associated with more advanced disease. Comparing cases with local disease to those with advanced disease, we found slightly elevated ORs for advanced disease in those subjects with the TaqI T allele or poly(A) L allele but these results were not statistically significant. These trends are consistent with the finding of Ingles et al. [23] that the poly(A) L allele was associated with more advanced disease. In contrast, Taylor et al. [22] found no association of the T allele with more advanced disease and, in a case-control study of blacks only, Ingles et al. [30] found no association of the poly(A) genotype alone with disease stage.

The functional consequences of the various polymorphisms in the VDR gene remain largely undetermined, hindering understanding of the potential link between VDR polymorphisms and prostate cancer. Morrison et al. [19] found associations between several VDR RFLPs (BsmI, ApaI, and EcoRV) and circulating levels of osteocalcin, a vitamin D–responsive product. In trying to determine a molecular basis for this physiologic finding, Morrison et al. [20] showed that the haplotype containing the long poly(A) allele demonstrates substantially less reporter gene activity in a minigene construct of the 3′-UTR, suggesting decreased gene transcription or decreased mRNA stability. Additionally, Ma et al. [29] recently found that the BB genotype is significantly associated with higher 1,25-D levels. This finding differs from an earlier report of no effect of BsmI or TaqI polymorphisms on serum 1,25-D levels [31]. Carling et al. [32] found that patients with the TaqI TT genotype demonstrate significantly lower *VDR* mRNA levels in parathyroid tumor samples than those patients with the tt genotype. However, other studies have found no effect of VDR polymorphisms on levels of VDR mRNA or on mRNA stability [33–35].

In summary, we found no statistically significant association of previously identified VDR polymorphisms and prostate cancer risk. Without a clearer understanding of what, if any, functionally significant polymorphisms exist in the VDR, it is difficult to establish a causal pathway by which VDR alleles affect cancer risk.

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